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(54) CCR2-64I, POLYMORPHIC VARIANT OF THE HUMAN CCRS RECEPTOR AND ITS USE IN THE DIAGNOSTIC AND TREATMENT OF ATHEROSCLEROSIS

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(57) The invention relates to the human CCR2 receptor (CCR2-64V), in particular a polymorphic variant form of the human CCR2 receptor (CCR2-64I), and its involvement as a risk factor in the development of atherosclerosis. Variant CCR2 polynucleotides and polypeptides are provided together with methods for their use including screening and diagnostic methods in the development of atherosclerosis.

Description Claims

CCR2-64I, POLYMORPHIC VARIANT OF THE HUMAN CCR2 RECEPTOR AND ITS USE IN THE DIAGNOSTIC AND TREATMENT OF ATHEROSCLEROSIS The present invention relates to the human CCR2 receptor, in particular a polymorphic variant form of the human CCR2 receptor, and its involvement as a risk factor in the development of atherosclerosis.

Atherosclerotic plaque rupture and resulting intracoronary thrombosis are thought to account for most acute coronary syndromes, such as unstable angina, myocardial infarctions as well as many cases of sudden cardiac death. These acute coronary syndromes represent a major cause of overall morbidity and mortality in the developed world. The current primary therapy for atherosclerotic disease is aggressive plasma cholesterol lowering and is dominated by use of the HMG-COA reductase inhibitors, the statins. Overall, 50% of patients with cardiovascular disease are hypercholesterolaemic.

However in many cases the effectiveness of such treatments may be severely compromised by, for example, late diagnosis of the condition. Thus a patient may already have advanced atherosclerotic plaques before treatment commences, reducing the prospect of a successful therapeutic outcome. In addition, early diagnosis can enable preventative measures to be put in place, for example changes in aspects of lifestyle and diet that are known non-genetic risk factors for the development of atherosclerosis.

Therefore it is clear that there is a need for the development of new and effective diagnostic methods and indeed for new and effective treatments for atherosclerosis and hypercholesterolaemia. However these new and effective treatments are dependent on the identification of new and useful gene and/or protein targets which have a demonstrated link to the disease. These targets can be isolated and screens developed in order to identify compounds useful in the treatment of atherosclerosis.

The present invention is based upon the finding that polymorphic variants of CCR2 receptor are associated with a lower incidence of atherosclerosis in humans. The invention provides CCR2 receptor encoding polynucleotides, including those having the said variant sequences, and the polypeptides encoded thereby. Such polynucleotides and polypeptides are useful in, for example, the development of diagnostic assays and kits. In a preferred embodiment the invention provides a process for diagnosing atherosclerosis or susceptibility to atherosclerosis in a subject related to expression or activity of a CCR2-64I polypeptide of SEQ D NO : 2 or a CCR2-64V polypeptide of SEQ ID NO : 4 in a subject comprising: (a) determining the presence or absence of a SNP in codon 64 of the polynucleotide encoding the CCR2 receptor in the genome of said subject, said SNP resulting in the production of a CCR2-64I polypeptide of SEQ ID NO : 2 and/or (b) analyzing for the presence or amount of the CCR2-64I polypeptide of SEQ ID NO : 2 or the CCR2-64V polypeptide of SEQ ID NO : 4 in a sample derived from said subject.

In a further aspect the invention concerns the use of a compound selected from: (a) a CCR2-64V

polypeptide or fragments thereof; (b) a compound which inhibits the activity of a CCR2-64V polypeptide, (c) a compound which inhibits the dimerisation of CCR2-64V polypeptides; (c) a polynucleotide encoding a CCR2-64I polypeptide, for the manufacture of a medicament for treating: (i) atherosclerosis; or (ii) hypercholesterolaemia.

Prevention of disease or early therapy of disease is a highly desirable goal of health management organizations, health care providers, and of course the patients themselves. Early therapeutic intervention can lead to prevention, improved outcome and/or reduced length and cost of treatment. Such early intervention necessitates the accurate identification of patients at risk from a particular disease. Traditionally patients at risk have been identified according to phenotypic parameters (frequently after some phenotypic abnormality has manifested itself) or in some cases genetically if they are members of a family with a history of the disease condition. However recently it has become possible to accurately identify patients at risk at a much earlier stage and without the need to depend on phenotypic cues or family history. All that is required is a sample of the patient's blood, for example, which can then be genotyped to determine whether the individual carries a particular genetic polymorphism know to be associated with a particular disease state.

Such genotyping obviously requires knowledge of an association between a genetic polymorphism and a disease state. The determination of such associations is presently an active area of research.

As herein used the term genetic polymorphism is defined as a naturally occurring variation in the DNA sequence of an organism which may or may not result in phenotype variation.'Phenotype'may be defined as the combined physical characteristics of the organism and includes (but not exclusively) disease states in humans. Polymorphisms can be divided into two broad classes: single base substitutions (also known as single nucleotide polymorphisms or SNPs), and deletion/insertion events.

An SNP occurs when a specific nucleotide position within the DNA sequence has two or more states in the population under study, ie a different nucleotide may be present at the given position when different individuals are compared. SNPs can occur within genes (where'gene'is defined as the entire coding and regulatory regions giving rise to a specific protein). Such intragenic polymorphisms may or may not directly affect gene or protein function. SNPs may also lie outside genes (extragenic).

Deletion/insertion polymorphisms occur when one or more nucleotides is absent in one individual when compared to another. The most common type of insertion/deletion polymorphism used in genetic analysis is the tandem repeat sequence, where a specific stretch of DNA within the genome consists of a tandemly repeated motif. Such sequences show variability (polymorphism) in the number of repeats, resulting in different lengths of DNA fragment in different individuals. Tandem repeat polymorphisms can be divided into two categories, depending on the number of nucleotides comprising the repeat unit (n). The two classes are variable number of tandem repeat loci (VNTRs) where n>4 and simple tandem repeat loci (STRs) where N&#x0;LT;5. Both VNTRs and STRs can be used for genetic association studies. Tandem repeats typically lie outside genes but can also occur within genes.

Both intra-and extragenic polymorphisms can be used for the identification of genetic associations with phenotype. An intragenic polymorphism may have a direct influence on phenotype by altering the level of gene expression or the structure of the resultant protein. Alternatively, an intragenic or extragenic polymorphism of no direct functional consequence may be physically linked to a second polymorphism which is of functional significance, allowing a test for association with a phenotype indirectly, in the absence of any knowledge of the functional variant itself.

In addition to the use of genetic polymorphisms for the identification of patients at risk, such genotypic knowledge can be used to select patients groups for clinical trial studies, and also to interpret the results of such trials. Essentially the statistical power of clinical trial studies to detect efficacy of a therapeutic agents can be improved if appropriate knowledge of prognostic factors that can influence response to therapy is included as part of the study design.

In so far that genetic polymorphisms can be used as prognostic factors in clinical studies, the knowledge and assay of such polymorphisms has the potential of making some of these studies more cost effective. This is true if the inclusion of genetic prognostic factors translates in equivalent statistical power to detect efficacy with the smaller number of patients, thus decreasing the cost of a given study.

The particular SNP to which the present invention is concerned is found in the gene encoding the CCR2 receptor, the receptor for the monocyte chemoattactant protein, MCP-1. MCP-1 induces functional responses through CCR2 receptor dimerization. The formation of receptor homodimers is a critical event linked to specific ligand activation of the chemokinereceptor (PNAS 96: 3628-33 (1999)). CCR2 homodimerization is then thought to trigger JAK (Janus associated tyrosine kinase) kinase recruitment for activation of the signaling pathway (J. Immun. 161: 805-13 (1998)).

The SNP in the CCR2 polypeptide is a valine to isoleucine substitution at residue 64 which is located within the first transmembrane domain of CCR2. The amino acid sequence of this polymorphic variant polypeptide, herein termed CCR2-64I, is given in SEQ ID NO : 2. The polymorphic variant polynucleotide that encodes the CCR2-64I polypeptide of SEQ ID NO : 2 is shown in SEQ ID NO: 1. Thus there is a G to A transition at position 190 which alters a valine encoding GTC codon to an isoleucine encoding ATC codon.

The amino acid sequence of the wild-type CCR2 polypeptide sequence, herein termed CCR2-64V, is given in SEQ ID NO : 4. The polynucleotide encoding CCR2-64V is shown in SEQ ID NO : 3.

The CCR2-64I polymorphism occurs at an allele frequency of 10-25%, depending on the ethnic group of the population. Mellado et al (1999) Nature 400: 723- 24, recently reported that CXCR4 can dimerize with the CCR2-64I mutant, but not with wild-type CCR2-64V, indicating that Val 64 may be critical for dimer stabilization.

It should be noted here that the CCR2 receptor occurs in two alternative splice forms-CCR2a and CCR2b. The CCR2 polypeptide sequences shown in SEQ ID NO : 2 and SEQ ID NO : 4 are the CCR2b form. The

CCR2a and b forms are identical up to and including residue 313, but then the CCR2a sequence continues: SLFHIALGCRIAPLQKPVCGGPGVRPGKNVKVTTQGLLDGRGKGKSIGRAPEASL QDKEGA-COOH; whilst the CCR2b continues: RYLSVFFRKHITKRFCKQCPVFYRETVDGVTSTNTPSTGEQEVSAGL-COOH Obviously the polymorphism at amino acid residue position 64 occurs in both splice- forms. It is understood that all references to CCR2 polypeptides include both CCR2a and CCR2b forms, even though the invention is illustrated only by the CCR2b form.

The effect of the polymorphism was studied on the presence and extent of atherosclerotic plaque formation and on total plasma cholesterol levels in individuals with a family history of coronary heart disease.

Thus a study of a selected human subject group demonstrated that the presence of the CCR2-I64 mutant was associated with lower risk of coronary artery calcification (CAC).

Thus in a first aspect the present invention relates to the use of the polynucleotide of SEQ ED NO: 1 or SEQ ID NO : 3, or polynucleotides derived therefrom, as diagnostic reagents for detecting polymorphisms in the associated CCR2 gene. Detection of the CCR2- 64V form of the CCR2 gene characterised by the polynucleotide of SEQ ID NO : 3 in the cDNA or genomic sequence, and which is associated with an increased risk of atherosclerosis, will provide a diagnostic tool that can predict or diagnose an atherosclerosis or susceptibility to atherosclerosis. Detection of the CCR2-64I form of the CCR2 gene provides a diagnostic tool that will be predictive of a reduced risk of atherosclerosis. The diagnostic assay for detection of these CCR2 nucleotide differences may be carried out by a variety of techniques well known in the art.

Nucleic acids for diagnosis may be obtained from a subject's cells, such as from blood, urine, saliva, tissue biopsy or autopsy material. The genomic DNA may be used directly for detection or it may be amplified ENZYMATICALLY by using PCR, preferably RT- PCR, or other amplification techniques prior to analysis. RNA or cDNA may also be used in similar fashion. Deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to labeled CCR2-64V or CCR2-64I nucleotide sequences.

Perfectly matched sequences can be distinguished from mismatched duplexes by RNase digestion or by differences in melting temperatures. DNA sequence difference may also be detected by alterations in the ELECTROPHORETIC mobility of DNA fragments in gels, with or without denaturing agents, or by direct DNA sequencing (see, for instance, Myers ET AL., Science (1985) 230: 1242). Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S I protection or the chemical cleavage method (see Cotton et aL, Proc Natl Acad Sci USA (1985) 85: 4397-4401).

Detection of abnormally decreased or increased levels of polypeptide or MRNA expression may also be used for diagnosing or determining susceptibility of a subject atherosclerosis. Decreased or increased expression can be measured at the RNA level using any of the methods well known in the art for the

quantitation of polynucleotides, such as, for example, nucleic acid amplification, for instance PCR, RT-PCR, RNase protection, Northern blotting and other hybridization methods. Assay techniques that can be used to determine levels of a protein, such as a polypeptide of the present invention, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include RADIOIMMUNOASSAYS, competitive-binding assays, Western Blot analysis and ELISA assays.

Thus in another aspect, the present invention relates to a diagonostic kit comprising: (a) a polynucleotide of the present invention, preferably the nucleotide sequence of SEQ ID NO: 3, SEQ ID NO: 1 or a fragment or an RNA transcript thereof; (b) a nucleotide sequence complementary to that of (a); (c) a polypeptide of the present invention, preferably the polypeptide of SEQ ID NO : 4, SEQ ID NO : 2 or a fragment thereof; or (d) an antibody to a polypeptide of the present invention, preferably to the polypeptide of SEQ ID NO : 4 or SEQ ID NO : 2.

In a preferred embodiment the diagnostic assay for the CCR2-64I allele involves the detection of an SNP in codon 64 wherein the SNP is a G to A substitution at nucleotide 190.

In a further embodiment the invention relates to the use of a method of assaying for the presence or absence of the polynucleotides OF SEQ ID NO : 3 and SEQ ID NO : 1 for: a) predicting the likelihood of developing atherosclerosis; b) predicting and responding to the progression of the atherosclerotic condition; c) predicting and responding to reaction to drug treatment; or d) predicting disease outcome. in an individual.

In a particularly preferred embodiment the reaction to drug treatment is an adverse event to a therapeutic compound useful in the treatment of atherosclerosis.

In a further aspect the invention relates to the use of a method of assaying for the presence or absence of the polynucleotides of SEQ ID NO : 3 and SEQ ID NO : 1 for the selection of patient groups for conducting clinical trials concerning therapeutic compounds with potential for use in the treatment of atherosclerosis.

In another aspect, the present invention provides for a method of screening compounds to identify those which inhibit the function of the CCR2-64V polypeptide. Such inhibition may be achieved : a) by inhibiting the activity of the CCR2-64V receptor by blocking (antagonising) binding of the MCP-1 ligand or by blocking the normal CCR2-64V stimulated signalling in some other way, for example by using an allosteric inhibitor; or b) by preventing dimerisation of the CCR2-64V receptor which is an important step in CCR2-64V signalling.

In general, agonists or antagonists identified using such a screen may be employed for therapeutic and prophylactic purposes for such diseases as atherosclerosis. Compounds may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. Such agonists, antagonists or inhibitors so- identified may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of the polypeptide ; or may be structural or functional

mimetics thereof (see Coligan et al., Current Protocols in Immunology 1 (2): Chapter 5 (1991)).

The screening method may simply measure the binding of a candidate compound to the polypeptide, or to cells or membranes bearing the polypeptide, or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound.

Alternatively, the screening method may involve competition with a labeled competitor.

Further, these screening methods may test whether the candidate compound results in a signal generated by activation or inhibition of the polypeptide, using detection systems appropriate to the cells bearing the polypeptide. Inhibitors of activation are generally assayed in the presence of a known agonist and the effect on activation by the agonist by the presence of the candidate compound is observed. In the case of inhibiting dimerisation, the screen may involve measuring the ability of a compound to inhibit the binding of one CCR2-64V polypeptide to another CCR2-64V polypeptide.

Constitutively active polypeptides may be employed in screening methods for inverse agonists or inhibitors, in the absence of an agonist or inhibitor, by testing whether the candidate compound results in inhibition of activation of the polypeptide. Further, the screening methods may simply comprise the steps of mixing a candidate compound with a solution containing a polypeptide of the present invention, to form a mixture, measuring CCR2-64V activity in the mixture, and comparing the CCR2-64V activity of the mixture to a standard. Fusion proteins, such as those made from Fc portion and CCR2-64V polypeptide, as hereinbefore described, can also be used for high-throughput screening assays to identify antagonists for the polypeptide of the present invention (see D. Bennett et AL., J Mol Recognition, 8: 52-58 (1995); and K. Johanson ET AL., J Biol Chem, 270 (16): 9459-9471 (1995)).

The polynucleotides, polypeptides and antibodies to the polypeptide of the present invention may also be used to configure screening methods for detecting the effect of added compounds on the production OF MRNA and polypeptide in cells. For example, an ELISA assay may be constructed for measuring secreted or cell associated levels of polypeptide using monoclonal and polyclonal antibodies by standard methods known in the art. This can be used to discover agents which may inhibit or enhance the production of polypeptide (also called antagonist or agonist, respectively) from suitably manipulated cells or tissues.

Thus in one aspect the invention relates to a method for screening to identify compounds that inhibit the activity, dimerisation or level of a polypeptide selected from the group consisting of : (i) a polypeptide comprising a polypeptide having the amino acid sequence OF SEQ ID NO : 4; and (ii) a polypeptide having the amino acid sequence OF SEQ ID NO : 4, comprising a method selected from the group consisting of : (a) measuring or, detecting, quantitatively or qualitatively, the bindingof a candidate compound to the polypeptide (or to the cells or membranes expressing the polypeptide) or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound; (b) measuring the competition of binding of a candidate compound to the polypeptide (or to the cells or membranes expressing the polypeptide) or a fusion protein thereof in the presence of a labeled

competitior; (c) testing whether the candidate compound results in a signal generated by activation or inhibition of the polypeptide, using detection systems appropriate to the cells or cell membranes expressing the polypeptide; (d) mixing a candidate compound D WITH a solution containing the polypeptide, to form a mixture, measuring activity of the polypeptide in the mixture, and comparing the activity of the mixture to a control mixture which contains no candidate compound; or (e) detecting the effect of a candidate compound on the production OFMRNA encoding said polypeptide or said polypeptide in cells, using for instance, an ELISA assay.

Examples of potential polypeptide antagonists include antibodies or, in some cases, oligonucleotides or proteins which are closely related to the ligands, substrates, receptors, enzymes, etc., as the case may be, of the polypeptide, e. g., a fragment of the ligands, substrates, receptors, enzymes, etc.; or small molecules which bind to the polypeptide of the present invention but do not elicit a response, so that the activity of the polypeptide is prevented.

Thus, in another aspect, the present invention relates to a screening kit for identifying agonists, antagonists, ligands, receptors, substrates, enzymes, etc. for polypeptides of the present invention; or compounds which decrease or enhance the production of such polypeptides, which comprises: (a) a polypeptide of the present invention; (b) a recombinant cell expressing a polypeptide of the present invention; (c) a cell membrane expressing a polypeptide of the present invention; or (d) antibody to a polypeptide of the present invention; which polypeptide is preferably that OF SEQ ID NO : 4.

It will be readily appreciated by the skilled artisan that a polypeptide of the present invention may also be used in a method for the structure-based design of an agonist, antagonist or inhibitor of the polypeptide, by: (a) determining in the first instance the three-dimensional structure of the polypeptide; (b) deducing the three-dimensional structure for the likely reactive or binding site (s) of an agonist, antagonist or inhibitor; (c) synthesing candidate compounds that are predicted to bind to or react with the deduced binding or reactive site; and (d) testing whether the candidate compounds are indeed agonists, antagonists or inhibitors.

It will be further appreciated that this will normally be an iterative process.

The invention further relates to antagonist compounds identified using the screening methods of the invention and to their use in the manufacture of a medicament for the treatment of atherosclerosis. The invention also relates to processes for manufacturing the compounds that have been identified in the screen.

In a further aspect, the present invention provides methods of treating abnormal conditions such as, for instance, atherosclerosis, related to an excess of CCR2-64V polypeptide activity.

If the activity of the CCR2-64V polypeptide is in excess, several approaches are available. One approach comprises administering to a subject in need thereof an inhibitor compound (antagonist) as hereinabove described, optionally in combination with a pharmaceutically acceptable carrier, in an amount effective

to inhibit the function of the CCR2-64V polypeptide, such as, for example, by blocking the binding of ligands, homologous CCR2-64V polypeptides (dimerisation), substrates, receptors, enzymes, etc., or by inhibiting a second signal, and thereby alleviating the abnormal condition. In another approach, soluble forms of the polypeptides still capable of binding the ligand, substrate, enzymes, receptors, etc. in competition with endogenous polypeptidemay be administered. Typical examples of such competitors include fragments of the CCR2-64V polypeptide.

In still another approach ; expression of the gene encoding endogenous CCR2- 64V polypeptide can be inhibited using expression blocking techniques. Known such techniques involve the use of antisense sequences, either internally generated or externally administered (see, for example, O'Connor, J Neurochem (1991) 56: 560 in Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). Alternatively, oligonucleotides which form triple helices ("triplexes") with the gene can be supplied (see, for example, Lee et AL., Nucleic Acids Res (1979) 6: 3073; Cooney et al., Science (1988) 241: 456; Dervan et AL., Science (1991) 251: 1360).

These oligomers can be administered per se or the relevant oligomers can be expressed in vivo. Synthetic antisense or triplex oligonucleotides may comprise modified bases or modified backbones. Examples of the latter include methylphosphonate, phosphorothioate or peptide nucleic acid backbones. Such backbones are incorporated in the antisense or triplex oligonucleotide in order to provide protection from degradation by nucleases and are well known in the art. Antisense and triplex molecules synthesised with these or other modified backbones also form part of the present invention.

In addition, expression of the human CCR2-64V polypeptide may be prevented by using ribozymes specific to the human CCR2-64V MRNA sequence. Ribozymes are catalytically active RNAs that can be natural or synthetic (see for example Usman, N, et al., Curr. Opin. STRUCT. Biol (1996) 6 (4), 527-33.) Synthetic ribozymes can be designed to specifically cleave human CCR2-64V mRNAs at selected positions thereby preventing translation of the human CCR2-64V mRNAs into functional polypeptide. RIBOZYMES may be synthesised with a natural ribose phosphate backbone and natural bases, as normally found in RNA molecules. Alternatively the RIBOSYMES may be synthesised with non-natural backbones to provide protection from ribonuclease degradation, for example, 2&#x0;-O-METHYL RNA, and may contain modified bases.

In a further aspect, the present invention provides for pharmaceutical compositions comprising a therapeutically effective amount of a polypeptide, such as the soluble form of a polypeptide of the present invention, agonist/antagonist peptide or small molecule compound, in combination with a PHARMACEUTICALLY acceptable carrier or excipient. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention. Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

The composition will be adapted to the route of administration, for instance by a systemic or an oral route. Preferred forms of systemic administration include injection, typically by intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can be used. Alternative means for systemic administration include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if a polypeptide or other compounds of the present invention can be formulated in an enteric or an encapsulated formulation, oral administration may also be possible. Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels, and the like.

The dosage range required depends on the choice of peptide or other compounds of the present invention, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. Suitable dosages, however, are in the range of 0.1-100 LLGLKG of subject. Wide variations in the needed dosage, however, are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection.

Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

Polypeptides used in treatment can also be generated endogenously in the subject, in treatment modalities often referred to as"gene therapy"as described above.

Thus, for example, cells from a subject may be engineered with a polynucleotide, such as a DNA or RNA encoding the CCR2-64I polypeptide, to replace or exist alongside the polynucleotide encoding the CCR2-64V polypeptide. Thus the entire CCR2-64V polypeptide expression is eliminated or significantly reduced. Such reduced expression of the CCR2-64V polypeptide is therapeutically beneficial with regard the development or susceptibility to atherosclerosis. Such polynucleotides encoding the CCR2-64I polypeptide can be introduced into cells ex-vivo by the use of a retroviral plasmid vector. The cells are then introduced into the subject.

Thus in a further aspect the invention provides for the use of a compound selected from : (a) a CCR2-64V polypeptide or fragments thereof ; (b) a CCR2-64I polypeptide or fragments thereof; (c) a compound which inhibits the activity of a CCR2-64V polypeptide ; (c) a compound which inhibits the dimerisation of CCR2-64V polypeptides; (d) a polynucleotide encoding a CCR2-64V polypeptide; (e) a polynucleotide encoding a CCR2-64I polypeptide, for the manufacture of a medicament for treating: (i) atherosclerosis; or (ii) hypercholesterolaemia.

Preferably the medicament comprises an isolated polypeptide which comprises a polypeptide having at least 95% identity to the CCR2-64VPOLYPEPTIDE OF SEQ ID NO : 4, most preferably the isolated polypeptide OF SEQ ID NO : 4. In another embodiment the medicament comprises an isolated polypeptide which comprises a polypeptide having at least 95% identity to the CCR2-64IPOLYPEPTIDE

OF SEQ ID NO : 2, preferably the polypeptide OF SEQ ID NO : 2.

In a further embodiment the medicament comprises a soluble form of the CCR2- 64V polypeptide.

In a still further embodiment the medicament comprises a polynucleotide encoding a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO : 2, preferably a polynucleotide having at least 95% identity with the polynucleotide of SEQ ID NO: 1, most preferably the polynucleotide of SEQ ID NO: 1.

Such screens require CCR2-64V and CCR2-64I polypeptides and polynucleotides. The CCR2-64V and CCR2-64I polynucleotides of the invention may be obtained using standard cloning and screening techniques from a cDNA library derived from MRNA in cells of tissue (see for instance, Sambrook et AL., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. (1989)). Polynucleotides of the invention can also be obtained from natural sources such as genomic DNA libraries or can be synthesized using well known and commercially available techniques.

The polynucleotide sequence OF SEQ ID NO: 1 is a cDNA sequence that encodes the CCR2-64I polypeptide OF SEQ ID NO : 2. The polynucleotide sequence encoding the polypeptide of SEQ ID NO : 2 may be identical to the polypeptide encoding sequence of SEQ ID NO: 1 or it may be a sequence other than SEQ ID NO: 1, which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptide of SEQ ID NO : 2.

The polynucleotide sequence of SEQ ID NO : 3 is a cDNA sequence that encodes the CCR2-64V polypeptide of SEQ ID NO : 4. The polynucleotide sequence encoding the polypeptide of SEQ ID NO : 4 may be identical to the polypeptide encoding sequence of SEQ ID NO : 3 or it may be a sequence other than SEQ ID NO : 3, which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptide of SEQ ID NO : 4.

When polynucleotides of the present invention are used for the recombinant production of polypeptides of the present invention, the polynucleotide may include the coding sequence for the mature polypeptide, by itself, or the coding sequence for the mature polypeptide in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro-or prepro-protein sequence, or other fusion peptide portions. For example, a marker sequence that facilitates purification of the fused polypeptide can be encoded. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, INC.) and described in Gentz et al., Proc Natl Acad Sci USA (1989) 86: 821-824, or is an HA tag. The polynucleotide may also contain non-coding 5&#x0;AND 3'sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize MRNA.

Recombinant polypeptides of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems.

Accordingly, in a further aspect, the present invention relates to expression systems comprising a polynucleotide or polynucleotides of the present invention, to host cells which are genetically engineered with such expression sytems and to the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention.

Polynucleotides may be introduced into host cells by methods described in many standard laboratory manuals, such as Davis et AL, Basic Methods in Molecular Biology (1986) and Sambrook ET AL. (IBID). Preferred methods of introducing polynucleotides into host cells include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, TRANSVECTION, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as STREPTOCOCCI, STAPHYLOCOCCI, E. COLI, STREPTOMYCES AND BACILLUS SUBTILIS cells; fungal cells, such as yeast cells and Aspergillus cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS, HeLa, C127,3T3, BHK, HEK 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used, for instance, chromosomal, episomal and virus-derived systems, e. g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector that is able to maintain, propagate or express a polynucleotide to produce a polypeptide in a host may be used. The appropriate polynucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook et AL., (IBID). Appropriate secretion signals may be incorporated into the desired polypeptide to allow secretion of the translated protein into the lumen of the endoplasmic reticulum, the periplasmic space or the extracellular environment. These signals may be endogenous to the polypeptide or they may be HETEROLOGOUS signals.

If a polypeptide of the present invention is to be expressed for use in screening assays, it is generally preferred that the polypeptide be produced at the surface of the cell.

In this event, the cells may be harvested prior to use in the screening assay. If the polypeptide is secreted into the medium, the medium can be recovered in order to recover and purify the polypeptide. If produced intracellularly, the cells must first be LYSED before the polypeptide is recovered.

Polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during intracellular synthesis, isolation and/or purification.

The invention will now be illustrated by the following examples.

Examples Example 1-Human subjects and study design 428 Caucasian individuals from the U. S., 176 females and 252 males tested for coronary artery calcification (CAC) using EBCT and plasma CLDL, triglycerides and HDL. People were selected to have a family history of coronary heart disease (at least on first degree relative with premature CHD) and absence of common risk factors. Active smokers uncontrolled hypertensives, obese, diabetic and hypercholesterolemic individuals were all excluded. The technique to measure atherosclerosis, electron beam computed tomography (EBCT), is a sensitive and specific measure of coronary artery calcification, and is valuable for non-invasive quantification of atherosclerotic plaque size (Circulation 93: 1951-53 (1996, Mayo Clin. Proc 71: 369-77 (1996) Results We compared the frequency of genetic types among subject that had CAC and those who did not (using an EBCT score of 20 as a threshold) (Table 1) Table 1: Gene frequency of the CCR2B genetic variant and CAC. CCR2 64 CAC-CAC+| X2 p-value |Prob CACl aa Relative] Women Ile 12. 6% 9.1% 0. 24 Val 87. 4% 90.9% 0.32 total214 66 0. 60 ns Men Ile 13. 3% 6.8% 0.48 Val 86. 7% 93.2% 0.78 total 256 1920.03 We observed that indeed the presence of the CCR2 I64 mutant was associated with lower risk of CAC. The trends were the same both in males and females, but results were statistically significant only among males ($p<0.03$).

In those subjects with EBCT scores >0, the effect of CCR2 genetic type on the extent of CAC was studied. The amino acid residue CCR2 64 was analysed using age, gender and BMI as covariates and it showed a significant association with EBCT score ($p<0.005$, not shown). Women carrying the CCR2-I64 mutant had on average 30% lower EBCT scores than Val carriers. In men the difference was 17% on average. This is consistent with the fact that CCR2-I64 carriers should have lower MCP-1 induced signal transduction and therefore less monocyte recruitment.

A significant effect OF CCR2I64 variant on plasma levels of CLDL was found in men ($p<0.05$). No effect was observed with levels of HDL nor on triglycerides (not shown).

Han and co-workers (ATVB 18: 1983-91 (1998) have shown that CCR2 expression in monocytes is greatly affected by LDL and that monocytes isolated from hypercholesterolemic patients show a two-fold increase in CCR2 expression (J Lipid Res 40: 1053-63 (1999). While LDL increases CCR2 expression, HDL induces inhibition of CCR2 monocyte expression. Our results suggest the possible existence of a feedback regulatory mechanism whereby signal transduction by CCR2 is sensed and has an effect on plasma cholesterol. If this is the case, a CCR2 antagonist would be expected to also modulate LDL

plasma levels. In conclusion, this study has shown that in humans, a mutant in the CCR2B gene (likely to be associated with lower signal transduction) results in: 1. a lower risk of developing atherosclerotic plaque 2. lower amounts of plaque where coronary artery disease is present 3. lower plasma levels of LDL cholesterol Example 2-Genotyping Genotyping : Allele specific amplification primers for the CCR2V64I polymorphism were used in conjunction with a common forward oligo. Two PCRs were conducted for each DNA sample (one for each Allele specific oligonucleotide) using the following conditions: 94°C for 40 seconds, 60°C for 30 seconds, 35 cycles in 20 PL reactions containing L OPMOLES of each primer, 1 U TaqExpress (GenPak Ltd.), 50 mM Tris. HCI pH9.1,16 mM ammonium sulphate, 3.5 mM MgC12, 150 UG/ML bovine serum albumin, plus 25-100NG of DNA. For each DNA, the two ASA reactions were run independently on 3% agarose gels and each lane scored for presence and absence of each allele. Allele specific oligos used were: CCR2V64IRC: 5'TTG CAG TTT ATT AAG ATG CGG AC, CCR2V64IRT : 5'TTG CAG TTT ATT AAG ATG CGG AT.

Common forward oligo: CCR2V64IF: 5'TAC CAA CGA GAG CGG TGA AGA AGT.

Sequence Information SEQ ID NO : 1
ATGCTGTCCACATCTCGTTCTCGGTTTATCAGAAATACCAACGAGAGCGGTGAAGAAGTCACCACCT
TTTTTTGATTATGA
TTACGGTGCTCCCTGTCATAAATTTGACGTGAAGCAAATTGGGGCCCAACTCCTGCCTCCGCTCTAC
TCGCTGGTGTTCA
TCTTTGGTTTTGTGGGCAACATGCTGGTCATCCTCATCTTAATAAACTGCAAAAAGCTGAAGTGCTT
GACTGACATTTAC &#x0;BR&#x0; &#x0;BR&#x0;
CTGCTCAACCTGGCCATCTCTGATCTGCTTTTTCTTATTACTCTCCCATTGTGGGCTCACTCTGCTGC
AAATGAGTGGGT
CTTTGGGAATGCAATGTGCAAATTATTCACAGGGCTGTATCACATCGGTTATTTTGGCGGAATCTTC
TTCATCATCCTCC
TGACAATCGATAGATACCTGGCTATTGTCCATGCTGTGTTTGCTTTAAAAGCCAGGACGGTCACCTT
TGGGGTGGTGACA
AGTGTGATCACCTGGTTGGTGGCTGTGTTTGCTTCTGTCCCAGGAATCATCTTTACTAAATGCCAGA
AAGAAGATTCTGT
TTATGTCTGTGGCCCTTATTTTCCACGAGGATGGAATAATTTCCACACAATAATGAGGAACATTTTGG
GGCTGGTCCTGC
CGCTGCTCATCATGGTCATCTGCTACTCGGGAATCCTGAAAACCCTGCTTCGGTGTCGAAACGAGA
AGAAGAGGCATAGG
GCAGTGAGAGTCATCTTCACCATCATGATTGTTTACTTTCTCTTCTGGACTCCCTATAACATTGTCAT
TCTCCTGAACAC
CTTCCAGGAATTCTTCGGCCTGAGTAACTGTGAAAGCACCAGTCAACTGGACCAAGCCACGCAGGT
GACAGAGACTCTTG
GGATGACTCACTGCTGCATCAATCCCATCATCTATGCCTTCGTTGGGGAGAAGTTCAGAAGGTATCT
CTCGGTGTTCTTC
CGAAAGCACATCACCAAGCGCTTCTGCAAACAATGTCCAGTTTTCTACAGGGAGACAGTGGATGGA
GTGACTTCAACAAA CACGCCTTCCACTGGGGAGCAGGAAGTCTCGGCTGGTTTATAA SEQ ID NO :

2 MLSTSRSRFIRNTNESGEEVTTFFDYDYGAPCHKFDVKQIGAQLLPPLYSLVFIFGFVGN
MLVILILINCKKLKCLTDIYLLNLAISDLLFLITLPLWAHSAANEWVFGNAMCKLFTGLY
HIGYFGGIFFIILLTIDRYLAIVHAVFALKARTVTFGWTSVITWLVAVFASVPGIIFTK
CQKEDSVYVCGPYFPRGWNNFHTIMRNILGLVLPLLIMVICYSGILKTLLRCRNEKKRHR
AVRVIFTIMIVYFLFWTPYNIVILLNTFQEFFGLSNCESTSQLDQATQVTETLGMTHCCI
NPIIYAFVGEKFRRYLSVFFRKHITKRFCKQCPVFYRETVDGVTSTNTPSTGEQEVSAGL SEQ ID NO :
3
ATGCTGTCCACATCTCGTTCTCGGTTTATCAGAAATACCAACGAGAGCGGTGAAGAAGTCACCACCT
TTTTTGATTATGA
TTACGGTGCTCCCTGTCATAAATTTGACGTGAAGCAAATTGGGGCCCAACTCCTGCCTCCGCTCTAC
TCGCTGGTGTTCA
TCTTTGGTTTTGTGGGCAACATGCTGGTCGTCCTCATCTTAATAAACTGCAAAAAGCTGAAGTGCTT
GACTGACATTTAC
CTGCTCAACCTGGCCATCTCTGATCTGCTTTTTCTTATTACTCTCCCATTGTGGGCTCACTCTGCTGC
AAATGAGTGGGT
CTTTGGGAATGCAATGTGCAAATTATTCACAGGGCTGTATCACATCGGTTATTTTGGCGGAATCTTC
TTCATCATCCTCC
TGACAATCGATAGATACCTGGCTATTGTCCATGCTGTGTTTGCTTTAAAAGCCAGGACGGTCACCTT
TGGGGTGGTGACA
AGTGTGATCACCTGGTTGGTGGCTGTGTTTGCTTCTGTCCCAGGAATCATCTTTACTAAATGCCAGA
AAGAAGATTCTGT
TTATGTCTGTGGCCCTTATTTTCCACGAGGATGGAATAATTTCCACACAATAATGAGGAACATTTTGG
GGCTGGTCCTGC
CGCTGCTCATCATGGTCATCTGCTACTCGGGAATCCTGAAAACCCTGCTTCGGTGTCGAAACGAGA
AGAAGAGGCATAGG
GCAGTGAGAGTCATCTTCACCATCATGATTGTTTACTTTCTCTTCTGGACTCCCTATAACATTGTCAT
TCTCCTGAACAC
CTTCCAGGAATTCTTCGGCCTGAGTAACTGTGAAAGCACCAGTCAACTGGACCAAGCCACGCAGGT
GACAGAGACTCTTG
GGATGACTCACTGCTGCATCAATCCCATCATCTATGCCTTCGTTGGGGAGAAGTTCAGAAGGTATCT
CTCGGTGTTCTTC
CGAAAGCACATCACCAAGCGCTTCTGCAAACAATGTCCAGTTTTCTACAGGGAGACAGTGGATGGA
GTGACTTCAACAAA CACGCCTTCCACTGGGGAGCAGGAAGTCTCGGCTGGTTTATAA SEQ ID NO :
4 MLSTSRSRFIRNTNESGEEVTTFFDYDYGAPCHKFDVKQIGAQLLPPLYSLVFIFGFVGN
MLVVLILINCKKLKCLTDIYLLNLAISDLLFLITLPLWAHSAANEWVFGNAMCKLFTGLY&#x0;BR&#x
0; HIGYFGGIFFIILLTIDRYLAIVHAVFALKARTVTFGWTSVITWLVAVFASVPGIIFTK
CQKEDSVYVCGPYFPRGWNNFHTIMRNILGLVLPLLIMVICYSGILKTLLRCRNEKKRHR
AVRVIFTIMIVYFLFWTPYNIVILLNTFQEFFGLSNCESTSQLDQATQVTETLGMTHCCI
NPIIYAFVGEKFRRYLSVFFRKHITKRFCKQCPVFYRETVDGVTSTNTPSTGEQEVSAGL

Description Claims

Claims 1. A process for diagnosing atherosclerosis or susceptibility to atherosclerosis in a subject related to expression or activity of a CCR2-64I polypeptide OF SEQ ID NO : 2 or a CCR2-64V polypeptide OF SEQ ID NO : 4 in a subject comprising: (a) determining the presence or absence of a SNP in codon 64 of the polynucleotide encoding the CCR2 receptor in the genome of said subject, said SNP resulting in the production of a CCR2-64I polypeptide of SEQ ID NO : 2 and/or (b) analyzing for the presence or amount of the CCR2-64I polypeptide of SEQ ID NO : 2 or the CCR2-64V polypeptide of SEQ ID NO : 4 in a sample derived from said subject.

2. A process according to claim 2 wherein the SNP in codon 64 of the polynucleotide is a G to A substitution at nucleotide 190.

3. The use of a method of assaying for the presence or absence of the polynucleotides of SEQ ID NO : 3 and SEQ ID NO : 1 for: a) predicting the likelihood of developing atherosclerosis; b) predicting and responding to the progression of the atherosclerotic condition; c) predicting and responding to reaction to drug treatment; or d) predicting disease outcome. in an individual.

4. Use according to claim 3 wherein the reaction to drug treatment is an adverse event to a therapeutic compound useful in the treatment of atherosclerosis.

5. The use of a method of assaying for the presence or absence of the polynucleotides of SEQ ID NO : 3 and SEQ ID NO : 1 for the selection of patient groups for conducting clinical trials concerning therapeutic compounds with potential for use in the treatment of atherosclerosis.

6. The use of a compound selected from : (a) a CCR2-64V polypeptide or fragments thereof ; (B) a CCR2-64I polypeptide or fragments thereof; (c) a compound which inhibits the activity of a CCR2-64V POLYPEPTIDQ (c) a compound which inhibits the dimerisation of CCR2-64V polypeptides; (d) a polynucleotide encoding a CCR2-64V polypeptide; (e) a polynucleotide encoding a CCR2-64I polypeptide, for the manufacture of a medicament for treating : (i) atherosclerosis; or (ii) hypercholesterolaemia.

7. The use according to claim 6 wherein the medicament is used in the treatment of atherosclerosis.

8. The use according to claim 6 wherein the medicament comprises an isolated polypeptide which comprises a polypeptide having at least 95% identity to the CCR2-64V polypeptide of SEQ BD NO : 4.

9. The use according to claim 6 wherein the medicament comprises an isolated polypeptide which comprises a polypeptide having at least 95% identity to the CCR2-64I polypeptide of SEQ ID NO : 2.

10. The use according to claim 8 wherein the isolated polypeptide is the CCR2-64V polypeptide of SEQ ID NO : 4.

11. The use according to claim 9 wherein the isolated polypeptide is the CCR2-64I polypeptide of SEQ ID NO : 2.

12. The use according to claim 6 wherein the medicament comprises a soluble form of the CCR2-64V polypeptide.

13. The use according to claim 6 wherein the medicament comprises a polynucleotide encoding a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO : 2.

14. The use according to claim 13 wherein the polynucleotide comprises a polynucleotide having at least 95% identity with the polynucleotide of SEQ ID NO: 1.

15. The use according to claim 14 wherein the polynucleotide has the polynucleotide sequence of SEQ ID NO: 1.

Claims 1. A process for diagnosing atherosclerosis or susceptibility to atherosclerosis in a subject related to expression or activity of a CCR2-64I polypeptide OF SEQ ID NO : 2 or a CCR2-64V polypeptide OF SEQ ID NO : 4 in a subject comprising: (a) determining the presence or absence of a SNP in codon 64 of the polynucleotide encoding the CCR2 receptor in the genome of said subject, said SNP resulting in the production of a CCR2-64I polypeptide of SEQ ID NO : 2 and/or (b) analyzing for the presence or amount of the CCR2-64I polypeptide of SEQ ID NO : 2 or the CCR2-64V polypeptide of SEQ ID NO : 4 in a sample derived from said subject.

2. A process according to claim 2 wherein the SNP in codon 64 of the polynucleotide is a G to A substitution at nucleotide 190.

3. The use of a method of assaying for the presence or absence of the polynucleotides of SEQ ID NO : 3 and SEQ ID NO : 1 for: a) predicting the likelihood of developing atherosclerosis; b) predicting and responding to the progression of the atherosclerotic condition; c) predicting and responding to reaction to drug treatment; or d) predicting disease outcome. in an individual.

4. Use according to claim 3 wherein the reaction to drug treatment is an adverse event to a therapeutic compound useful in the treatment of atherosclerosis.

5. The use of a method of assaying for the presence or absence of the polynucleotides of SEQ ID NO : 3 and SEQ ID NO : 1 for the selection of patient groups for conducting clinical trials concerning therapeutic compounds with potential for use in the treatment of atherosclerosis.

6. The use of a compound selected from : (a) a CCR2-64V polypeptide or fragments thereof ; (B) a CCR2-64I polypeptide or fragments thereof; (c) a compound which inhibits the activity of a CCR2-64V POLYPEPTIDQ (c) a compound which inhibits the dimerisation of CCR2-64V polypeptides; (d) a polynucleotide encoding a CCR2-64V polypeptide; (e) a polynucleotide encoding a CCR2-64I polypeptide,

for the manufacture of a medicament for treating : (i) atherosclerosis; or (ii) hypercholesterolaemia.

7. The use according to claim 6 wherein the medicament is used in the treatment of atherosclerosis.

8. The use according to claim 6 wherein the medicament comprises an isolated polypeptide which comprises a polypeptide having at least 95% identity to the CCR2-64V polypeptide of SEQ BD NO : 4.

9. The use according to claim 6 wherein the medicament comprises an isolated polypeptide which comprises a polypeptide having at least 95% identity to the CCR2-64I polypeptide of SEQ ID NO : 2.

10. The use according to claim 8 wherein the isolated polypeptide is the CCR2-64V polypeptide of SEQ ID NO : 4.

11. The use according to claim 9 wherein the isolated polypeptide is the CCR2-64I polypeptide of SEQ ID NO : 2.

12. The use according to claim 6 wherein the medicament comprises a soluble form of the CCR2-64V polypeptide.

13. The use according to claim 6 wherein the medicament comprises a polynucleotide encoding a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO : 2.

14. The use according to claim 13 wherein the polynucleotide comprises a polynucleotide having at least 95% identity with the polynucleotide of SEQ ID NO: 1.

15. The use according to claim 14 wherein the polynucleotide has the polynucleotide sequence of SEQ ID NO: 1.